3 = staining intensity strong, equal to positive control

4 = staining intensity greater than positive control.

Control slides used for comparison were paraffin-embedded MCF-7 40F cells (ATCC #HTB-22) for p53 and TSP-1. Microvascularization controls were paraffin-embedded tumor specimens showing high reactivity with anti-CD31 antibody.

Alternatively, TSP-1 expression was determined using image analysis (IA) techniques. Slides immunohistochemically stained for detection of TSP-1 expression as described above were analyzed using a CAS 200 image analysis system (Cell Analysis Systems, Lombard, IL) to quantitate the staining intensity of TSP-1 marker positive cells as described (*see* Figge *et al.*, 1991, *Amer. J. Pathol.* 139: 1213-1221 and Esteban *et al.*, 1993, *Amer. J. Clin. Pathol.* 99: 32-38). This analytical method uses a two-color system to sample image data using 2 solid-state video cameras, each with its own optical filter, mounted on a light microscope. Video signals are sent to an image capture board, which samples and digitizes the analog signal. The digital value of the signal sample is proportional to the amplitude of the video signal and is stored in an interactive computer. Measurements are obtained from calibrated conversion of pixel information from the video image.

For IA of TSP-1 expression in breast cancer tumor samples, the instrument was set at threshold values optimized to distinguish between cell membrane, nuclear and cytosolic portions of the stained image, and the zero pixel set-point adjusted using a tumor section stained with an isotype-matched irrelevant (*i.e.*, unrelated) antibody. At least 10 fields of positive area on the slide were scanned for each tumor specimen. Video values were converted to the product of the positive areas and positively-stained areas, expressed as optical density (O.D.) Units using instrument software. Antigen preservation control was evaluated using vimentin staining (1:200 antibody dilution, obtained from Dako, Carpenteria, CA). The results of IA were consistent with results obtained by visual analysis of the stained tumor sections.

The values of staining intensity related to "positive" or "negative" predicted outcomes were determined based on univariate analysis of the markers on survival, using a training subset (n=42) for which survival data were known. Immunohistochemistry (IHC) scores were assigned

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based on the product of the percentage of cells positive in the sample times (1 + intensity of staining), using the staining intensity scale described above. Tissue sections with immunodetectably nuclear p53 observed in more than 5% of the cells with 2+ staining intensity (corresponding to an IHC value > 15) were considered positive. (It is noted that the presence of nuclear-located p53 is used as a marker for mutant p53, consistent with the difference in cellular location of mutant p53 versus wildtype known in the prior art (Hall & Lane, 1994, *J. Pathol.* 172: 1-4). For IA of TSP-1 expression, positive sections were determined to have a value of >30 O.D. For angiogenesis, microvessels were counted in the region of greatest vessel density over at least 10 fields; samples designated as positive had > 70 vessels per field. Statistics, including Fischer's exact test and unpaired one-tailed t test were performed using a software program (GraphPad Software, v2.05, San Diego, CA) to compare values for each markers' incidence and intensity of expression as a function of histological progression.

The results of these assays are shown in Tables I and II. Table I presents the results for the tested markers based on a dichotomy of invasive *versus* non-invasive ductal breast carcinoma (as determined by pathological examination of breast tumor samples and registry information provided for each sample), while Table II shows the difference in staining patterns observed for the 4 histological subsets studied. These results show that highly significant changes in all three of the tested markers were observed in the transition from non-invasive to invasive disease. The frequency of nuclear p53 localization and microvascularization were found to be increased (> 5-fold) significantly ($p<0.0001$) in invasive tumor tissue, while the frequency of thrombospondin 1 expression decreased (> 5-fold) significantly ($p < 0.0001$) in these tumor samples.

When the tumor samples are further distinguished based on four (rather than two) subsets of morphological and histochemical criteria, additional differences were detected. As shown in Table II, frequency of nuclear localization of p53 increased significantly ($p=0.006$) in a comparison between low-grade and high-grade ductal carcinoma *in situ* (DCIS), even though both subsets are non-invasive. In these assays, nuclear p53 staining was not detected in any of the low-grade DCIS samples, while 31% (6/22) of the high-grade DCIS samples showed positive staining.

For the transition between high-grade (but non-invasive) DCIS to frankly invasive ductal

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carcinoma with negative lymph nodes, only the decline in TSP-1 expression was significant ($p < 0.002$), with the frequency of TSP-1 expression declining from 82% (18/22 samples) to 32% (6/19 samples). In addition, the transition from invasive ductal carcinoma without lymph node metastasis to invasive disease accompanied by lymph node metastasis showed significant changes in p53 nuclear localization, TSP-1 expression and microvascularization. The incidence of samples with p53 nuclear localization in tumor samples comprising metastatic cancer increased from 47% (9/19 samples) to 82% (14/17 samples) ($p=0.041$), the incidence of samples with high microvessel counts increased from 53% (10/19 samples) to 100% (17/17 samples) ($p=0.001$), and the incidence of samples with pronounced TSP-1 staining decreased from 32% (6/19 samples) in tumor without lymph node involvement to 0% (0/17 samples) in tumors associated with metastasis-positive lymph nodes ($p=0.02$).

TABLE I

Marker Profile: Invasive *versus* Non-invasive Ductal Breast Carcinoma

	Percent Positive Markers Studied:		
Tumor Type	p53	TSP-1	Microvasc.
Non-invasive (n=48)	12	83	12
Invasive (n=36)	64	17	75
P value*	<0.0001	<0.0001	<0.0001

* = P value determined by unpaired one-tailed t-test.

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